FORM PTO-1390 (Modified) (REV 10-95) U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE

TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371

ATTORNEY'S DOCKET NUMBER: **5093**

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR: **Not Yet Assigned**

INTERNATIONAL APPLICATION NO.	INTERNATIONAL FILING DATE	PRIORITY DATE CLAIMED
PCT/US97/13321	**23 July 1997**	**24 July 1996**

TITLE OF INVENTION
CHOLESTEROL SEPARATION AND FLUORESCENT ANALYSIS

09/230275

APPLICANT(S) FOR DO/EO/US
Debra Linn HICKS et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. ☒ This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. ☐ This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. ☐ This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. ☐ A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.
5. ☒ A copy of the International Application as filed (35 U.S.C. 371 (c) (2))
   - a. ☐ is transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ has been transmitted by the International Bureau.
   - c. ☒ is not required, as the application was filed in the United States Receiving Office (RO/US).
6. ☐ A translation of the International Application into English (35 U.S.C. 371(c)(2)).
7. ☐ A copy of the International Search Report (PCT/ISA/210).
8. ☒ Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371 (c)(3))
   - a. ☐ are transmitted herewith (required only if not transmitted by the International Bureau).
   - b. ☐ have been transmitted by the International Bureau.
   - c. ☐ have not been made; however, the time limit for making such amendments has NOT expired.
   - d. ☒ have not been made and will not be made.
9. ☐ A translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
10. ☐ An oath or declaration of the inventor(s) (35 U.S.C. 371 (c)(4)).
11. ☒ A copy of the International Preliminary Examination Report (PCT/IPEA/409).
12. ☐ A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371 (c)(5)).

Items 13 to 18 below concern document(s) or information included:

13. ☐ An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
14. ☐ An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
15. ☐ A **FIRST** preliminary amendment.
    A **SECOND** or **SUBSEQUENT** preliminary amendment.
16. ☐ A substitute specification.
17. ☐ A change of power of attorney and/or address letter.
18. ☐ Certificate of Mailing by Express Mail
19. ☐ Other items or information:

U.S. APPLICATION NO. (IF KNOWN, SEE 37 CFR	INTERNATIONAL APPLICATION NO.	ATTORNEY'S DOCKET NUMBER
Not Yet Assigned	**PCT/US97/13321**	**5093**

20. The following fees are submitted:.				CALCULATIONS	PTO USE ONLY
BASIC NATIONAL FEE (37 CFR 1.492 (a) (1) - (5)) :					
☐ Search Report has been prepared by the EPO or JPO			**$840.00**		
☒ International preliminary examination fee paid to USPTO (37 CFR 1.482)			**$670.00**		
☐ No international preliminary examination fee paid to USPTO (37 CFR 1.482) but international search fee paid to USPTO (37 CFR 1.445(a)(2))			**$760.00**		
☐ Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2) paid to USPTO			**$970.00**		
☐ International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(2)-(4)			**$96.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =				**$670.00**	
Surcharge of **$130.00** for furnishing the oath or declaration later than ☐ 20 ☒ 30 months from the earliest claimed priority date (37 CFR 1.492 (e)).				**$130.00**	
CLAIMS	NUMBER FILED	NUMBER EXTRA	RATE		
Total claims	20 - 20 =	0	x $18.00	**$0.00**	
Independent claims	2 - 3 =	0	x $78.00	**$0.00**	
Multiple Dependent Claims (**check if applicable**).			☐	**$0.00**	
TOTAL OF ABOVE CALCULATIONS =				**$800.00**	
Reduction of 1/2 for filing by small entity, if applicable. Verified Small Entity Statement must also be filed (Note 37 CFR 1.9, 1.27, 1.28) (**check if applicable**).			☐	**$0.00**	
SUBTOTAL =				**$800.00**	
Processing fee of **$130.00** for furnishing the English translation later than ☐ 20 ☐ 30 months from the earliest claimed priority date (37 CFR 1.492 (f)). +				**$0.00**	
TOTAL NATIONAL FEE =				**$800.00**	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31) (**check if applicable**).			☐	**$0.00**	
TOTAL FEES ENCLOSED =				**$800.00**	
				Amount to be: refunded	$
				charged	$

☒ A check in the amount of **$800.00** to cover the above fees is enclosed.

☐ Please charge my Deposit Account No. in the amount of to cover the above fees. A duplicate copy of this sheet is enclosed.

☒ The Commissioner is hereby authorized to charge any fees which may be required, or credit any overpayment to Deposit Account No. **04-1425** A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

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SIGNATURE

Jerold I. Schneider
NAME

24,765
REGISTRATION NUMBER

January 22, 1999
DATE

CHOLESTEROL SEPARATION AND FLUORESCENT ANALYSIS

Related Applications

This application claims the priority of U.S. Provisional Application No. 60/022,354, filed July 24, 1996, which is incorporated herein by reference.

Field of the Invention

The present invention relates to reagents and methods for the quantitative determination of cholesterol and cholesterol esters in serum lipoproteins. It has particular application in the medical and laboratory diagnostic fields where it is necessary to perform testing and analysis of biological and chemical substances.

Background of the Invention

The relationship of HDL Cholesterol to coronary heart disease was reported by D.P. Barr et al., in an article entitled: "Protein-lipid Relationships in Human Plasma, Am. J. Med., 11:480-493 (1951) and by G.J. Miller and N.E. Miller, in an article entitled: "Plasma-High Density-Lipoproteins Concentration and Development of Ischemic Heart Disease," Lancet, 1:16-19 (1976). The work of W.P. Castelli et al., as reported in "HDL Cholesterol and Other Lipids in Coronary Heart Disease - The Cooperative Lipoprotein Phenotyping Study," Circulation, 55(5):767-772 (1977) focused attention on HDL cholesterol assessment as the definitive laboratory test in determining the risk of coronary heart disease.[1] The cholesterol content of the lipoprotein fractions has been determined by ultracentrifugation, selective precipitation, and electrophoresis on several media.[2]

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1 Other works that discuss HDL cholesterol assessment as the definitive laboratory test in determining the risk of coronary heart disease include W.B. Kannel et al., "Serum Cholesterol, Lipoproteins, and the Risk of Coronary Heart Disease," Ann. Inter. Med. 74(1):1-12 (1971); T. Gordon et al., "High Density Lipoprotein As a Protective Factor Against Coronary Heart Disease," The Framingham Study, Am. J. Med., 62:707-714 (1977); and R.S. Galen, "HDL Cholesterol, How Good a Risk Factor," Diag. Med. 39-58, Nov/Dec (1979).

2 O.F. Delalla, et al., "Ultracentrifugal Analysis of Serum Lipoprotein," Methods of Biochemical Analysis, Vol. 1. 459-478 (1954); M. Burstein et al., "Precipitation of chylomicrons and very low density lipoproteins from human serum with sodium lauryl sulfate," Life Science, 11:177-184 (1972);

Clinical laboratory measurement of the serum lipoproteins is primarily due to their predictive association with risk of coronary heart disease. Current practice guiding laboratory measurement of total serum cholesterol, triglycerides, HDL cholesterol and LDL cholesterol is derived from recommendations of expert panels convened by the National Cholesterol Education Program (NCEP). The expert panels considered epidemiological, clinical, and intervention studies in developing the recommendations for treatment decision cutpoints and recommended workup sequences for adults and children.

The clinical recommendations from NCEP panels direct clinical laboratories to perform measurements of total, HDL and LDL, cholesterol and triglycerides. The triglycerides are primarily associated with chylomicrons, very low density (VLDL) and intermediate density (IDL) lipoproteins thought to be atherogenic, but the association of triglycerides with risk of coronary heart disease in epidemiological studies is ambiguous. LDL, as the validated atherogenic lipoprotein based on its cholesterol content, is the primary basis for treatment decisions in the NCEP clinical guidelines.[3] The major protein component of LDL is apolipoprotein B100 (apoB) which has been measured previously by immunoassay. The common research method for accurate LDL cholesterol quantitation and the basis for the reference method is designated beta-quantification, beta referring to the electrophoretic term for LDL. The beta-quantification technique involves a combination of ultracentrifugation and chemical precipitation.[4] The beta-quantification method

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and S.A. Cobb et al., "Enzymic Determination of Cholesterol in Serum Lipoproteins Separated by Electrophoresis," Clin. Chem., 24(7):1116-1120 (1978), respectively.

[3] National Cholesterol Education Program, Second report of the expert panel on detection, evaluation and treatment of high blood cholesterol in adults (Adult Treatment Panel II), NCEP (1993).

[4] U.S. Department of Health and Human Services, "Lipid Research Clinics Program," Manual of Laboratory Operations, Second Edition, NIH Publication (1983); and J.D. Belcher et al., "Measurement of low density lipoprotein cholesterol concentration," Methods for Clinical Laboratory Measurement of Lipid and Lipoprotein Risk Factors, Washington D.C, AACC Press, 75-86 (1991).

gives a so-called "broad cut" LDL which includes the Lp(a) lipoprotein,[5] often referred to "lipoprotein little a". The National Cholesterol Education Program Lipoprotein Measurement Working Group (NCEP panel) in its "Recommendations for measurement of low density lipoprotein cholesterol" (NIH Publication In Press) concluded that alternative methods are needed for routine diagnostic use, preferably ones which directly separate LDL for cholesterol quantitation· One such direct method involves electrophoresis. Electrophoretic methods, reviewed in L.A. Lewis and J.J. Opplt, CRC Handbook of Electrophoresis, Vols. 1 and 2, Boca Raton, CRC Press, Inc. (1980), have a long history of use in qualitative and quantitative analysis of lipoproteins. Electrophoresis not only allows separation and quantitation of major lipoprotein classes, but, in addition, provides a visual display useful in detecting unusual or variant patterns. Agarose has been the preferred media for separation of whole lipoproteins, providing a clear background and convenience.[6] Early electrophoretic methods were, in general, considered useful for qualitative analysis but less than desirable for lipoprotein quantitation because of poor precision and large systematic biases compared to other methods as reported in an article by G.R. Warnick et al., "Lipoprotein quantification: An electrophoretic method compared with the lipid research clinics method," Clin. Chem, 28:2116-20 (1982).

Summary of the Invention

The present invention is for use in the quantitative determination of cholesterol and cholesterol esters in the lipoproteins of serum following electrophoresis. The invention is intended to be used for the assessment of the cholesterol content of the

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5 G. Utermann, "The mysteries of lipoprotein(a)," Science, 246:904-910 (1989); and J. Loscalzo, "Lipoprotein(a): a unique risk factor for atherothrombotic disease," Arteriosclerosis 10:672-679 (1990).

6 R.P. Nobe, "Electrophoretic separation of plasma lipoproteins in agarose gel," J. Lipid Res. 9:693 (1968); F.T. Lindgren et al., "A comparison of simplified methods for lipoprotein quantitation using the analytic ultracentrifuge as a standard," Lipids, 12:278 (1977); D. Conlon et al., "Quantitative determination of high-density lipoprotein cholesterol by agarose gel electrophoresis updated," Clin. Chem., 24:227 (1979); and N.M. Papadopoulos, "Hyperlipoproteinemia phenotype determination by agarose gel electrophoresis updated," Clin. Chem., 24:227-229 (1978).

high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL). The methods and reagents of the invention provide for inexpensive, quick and efficient fluorescent analysis of the cholesterol content of electrophoresed lipoprotein bands.

The present system and invention separates the major lipoprotein classes using electrophoresis. The alpha band which migrates the farthest toward the corresponds to HDL. The next band, pre-beta, corresponds to VLDL, and the slowest moving beta band corresponds approximately to LDL. If a band appears between alpha and the origin, with fast pre-beta mobility, it corresponds to LDL and should be quantitated with the LDL band. This band may not be observed in every specimen. Chylomicrons, if present, remain at the origin.

Upon completion of electrophoresis the lipoprotein bands are stained with enzymic reagent of the present invention, so that the bands' cholesterol content can be quantitated by fluorometric densitometry. The reagent's active ingredients, and the direction of the reaction when the reagent's pH is 7.5-9.5 are shown as follows:

$$\text{Cholesterol Ester} \xrightleftharpoons[]{\textbf{Cholesterol Esterase}} \text{Cholesterol + Fatty Acid}$$

$$\text{Cholesterol} + \textbf{NAD+} \xrightleftharpoons[]{\textbf{Cholesterol Dehydrogenase}} \text{Cholesterone + NADH +H+}$$

* The active ingredients of the reagent are shown in bold.

The amount of NADH produced is directly proportional to the amount of cholesterol and cholesterol esters originally present in the sample. The relative percent cholesterol in each fraction is obtained by scanning in the fluorescent mode using a scanning densitometer, which detects the emissions from excited NADH molecules.

Thus, the invention broadly encompasses a reagent for staining lipoproteins for fluorometric analysis of electrophoretic bands, said reagent comprising enzymic components, and an oxidizing agent component.

The invention further encompasses a method for fluorescent analysis of the cholesterol content of high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL) on a electrophoretic plate, the method comprising:

(1) separating the lipoproteins electrophoretically;

(2) staining the separated proteins with a reagent comprising a enzymic component and an oxidizing agent component; and

(3) exciting the stained proteins with an appropriate wavelength of electromagnetic radiation; and

(4) scanning the exited proteins with a densitometer with fluorometric capabilities.

Detailed Description of the Invention

Any conventional electrophoresis instrument can be used to practice the present invention. In practicing the preferred embodiment described below, Helena Laboratories Corporation's Rapid ElectroPhoresis (REP®) and Rapid ElectroPhoresis 3 (REP® 3) instruments, which are commercially available, have been used. The REP® instrument and the use of this instrument are described in U.S. patent Nos. 4,810,348 and 4,909,920, which are hereby incorporated by reference. The REP® 3 instrument is similar to the REP® instrument, but includes an in situ fluorescent scanner. The REP® 3 instrument and the use of this instrument are described in commonly assigned copending application Serial Number _________ (Attorney Docket No. 5043), filed May 1, 1997, which is hereby incorporated by reference. The present invention will be explained in the context of a REP® 3 scanning electrophoresis apparatus marketed by Helena Laboratories Corporation.

In the preferred embodiment of the present invention fresh serum samples are electrophoresed using an agarose gel matrix or plate. The electrophoresis is carried out under native conditions. In addition to the agarose, the gel used includes quanidine hydrochloride, magnesium chloride or magnesium sulfate, sodium azide and other preservatives and an electrophoresis buffer, preferably sodium barbital with EDTA at a pH range of 7.6 to 9.6. In the preferred embodiment the sodium barbital has a pH of approximately 8.6. Utilizing a Helena Laboratories Corporation Rep® Electrophoresis system, the sample is electrophoresed at 225 volts

for 40 minutes at 12°C. It should be understood that other analyzers and systems will likely require different conditions for optimizing the assay.

After the electrophoresis step, the agarose plate is allowed to air dry for approximately 5 minutes. This air dry step is for the purpose of removing excess moisture from the surface of the gel before the reagent is applied. The plate is not completely dried in the this step. The stain or reagent of the present invention is then applied.

The concentration of the reactive ingredients of the reagent is as follows:

Cholesterol Esterase (Pseudomnae sp.)	4.5 U/mL*
Cholesterol Dehydrogenase (Nocardia sp.)	0.9 U/mL*
NAD	29 mM

*U/mL stands for units per milliliter in the reconstituted reagent (which is discussed below)

NAD is nicotinamide adenine dinucleotide, which is an oxidizing agent. More specifically, NAD is a proton-acceptor co-enzyme. In the reduced form NADH will fluoresce when excited with an appropriate wavelength of electromagnetic radiation.

Prior to use, the reagent's active ingredients are stored apart from the reagent's buffer. To reconstitute the active ingredients and prepare the reagent for use, the active ingredients in the amounts indicated above are dissolved in the inactive ingredient; 885 mM of Tricine Buffer having a pH in the range of 7.5-9.5. The active ingredients must be swirled gently until all of the active ingredients are dissolved. The reconstituted reagent is stable for approximately 2 to 4 hours at 2 to 6°C.

Once the reagent is applied to the electrophoresed plate, it is allowed to incubate with the cholesterol and cholesterol esters for approximately 3 minutes at 30°C. Following the incubation period the electrophoresed plate is dried at 54°C for 3 minutes. Again, it should be understood that other analyzers and systems will likely require different conditions for optimizing the assay. Thereafter, the plate is fluorometrically scanned. Quantitation of the fluorescent patterns has been obtained using the in situ scanner on Helena Laboratories Corporation's REP® 3, but any densitometer with fluorometric capability can be used. The fractions will

fluoresce at a peak excitation wavelength of 356nm, due to the presence of NADH, the reduced form of nicotinamide adenine dinucleotide.

Test Results

A total of 57 patients with total cholesterols* ranging from 124-200 mg/dL were tested using the above identified reagent and procedure with Helena Laboratories Corporation's REP® 3 instrument.[7] Twenty-three (23) of these specimens had Fast pre-beta with normal total cholesterol The following data was obtained:

N = 57

	RANGE ($\overline{X}$ + 2 SD)
HDL (%)	20.5 - 49.7
VLDL (%)	0 - 27.3
LDL+ pre-beta (%)	36.6 - 69.4

*Total Cholesterols were run using a Total Cholesterol method at 37°C.

Results

The REP® 3 Auto-Flur Cholesterol system separates the major lipoprotein classes. The alpha band which migrates the farthest toward the anode corresponds to HDL. The next band, pre-beta, corresponds to VLDL and the slowest moving beta band corresponds approximately to LDL. If a band appears between alpha and the origin, with fast pre-beta mobility, it corresponds to LDL and should be added to the LDL quantitation. This band may not be observed in every specimen. Chylomicrons, if present, remain at the origin.

Calculations

The Helena REP® 3 automatically calculates and prints the relative percent and the absolute values for each band when the specimen total cholesterol is entered.

7 Lipoprotein cholesterol values vary according to age and sex, and wide variations among different geographical locations and races have been reported. Therefore, it is essential that each laboratory establish its own expected range for its particular population.

Limitations

This method is intended for the separation and quantitation of lipoprotein classes. For those specimens which exhibit a band between alpha and the origin, with fast pre-beta mobility, it should be reported as part of the total LDL fraction. This band should not be reported as Lp(a) since sufficient clinical studies have not been done to substantiate this decision.

The system is linear to 400 mg/dL total cholesterol, or at least 250 mg/dL per band, with sensitivity to 2 mg/dL per band. Patient sample quantitations which exceed the linearity of the system should be diluted with saline and retested.

Interpretation of Results

Treatment decisions of the NCEP guidelines are based primarily on LDL cholesterol levels (Table 1). The risk factors considered in the classification scheme are age (males equal to or older than 45 years and females equal to or older than 55), family history of premature CHD, smoking, hypertension, and diabetes. Treatment is appropriate when LDL cholesterol is at or above the following cut points: all patients at or above 160 mg/dL, with two or more risk factors a value above 130 mg/dL.

HDL cholesterol is considered high risk at or below 35 mg/dL and counted as one of the risk factors in the classification scheme. An HDL cholesterol value above 60 mg/dL is considered protective and subtracts one from the total number of risk factors.

Treatment Decision Cut-Points

Total Cholesterol	
Desirable Blood Cholesterol	<200 mg/dL
Borderline-High Blood Cholesterol	200-239 mg/dL
High Blood Cholesterol	≥ 240 mg/dL

LDL-Cholesterol **Dietary Therapy**	**Initiation Level**	**LDL Goal**
Without CHD and fewer than 2 risk factors*	≥160 mg/dL	<160 mg/dL
Without CHD and with 2 or more risk factors*	≥130 mg/dL	<130 mg/dL
With CHD	>100 mg/dL	≤100 mg/dL

LDL Cholesterol **Drug Treatment**	**Initiation Level**	**LDL Goal**
Without CHD and fewer than 2 risk factors*	≥190 mg/dL	<16 mg/dL
Without CHD and with 2 or more risk factors*	≥160 mg/dL	<130 mg/dL
With CHD	≥130 mg/dL	≤100 mg/dL

HDL-Cholesterol	
Low HDL Cholesterol	<35 mg/dL
Protective HDL Cholesterol	>60 mg/dL

Triglycerides	
Desirable	<250 mg/dL
Borderline	250-500 mg/dL
Elevated	500-1000 mg/dL
Severely Elevated/Pancreatitis	>1000 mg/dL

Performance Characteristics

Precision

Within Run

A single patient sample was run a total of thirty times on one gel. This precision study gave CVs of less than 5%. No VLDL was observed in this sample.

N=30

	HDL%	LDL %
Mean	25.9	74.1
SD	0.8	0.8
CV	3.0%	1.0%

Between Run

Three different patient samples, representing low, middle and high LDL levels, were tested in replica showing excellent reproducibility.

		% HDL			% VLDL			% LDL + fast pre-beta (*if present)		
LDL Concentration (mg/dL)	n	mean	SC	CV	mean	SD	CV	mean	SC	CV
~90	80	26.2	1.6	6	11.2	1.5	13.3	62.6*	2.3	3.6
~120	80	42.6	2	4.6	18	1.8	10.1	39.4	2.4	6
~180	80	37	1.8	4.8	5.8	1	16.9	57.2*	2.1	3.7

Linearity and Sensitivity

Serial dilutions of an elevated cholesterol sample were made and tested by using the methods and reagent of the present invention. The linearity study showed that the system is linear to 400 mg/dL total cholesterol or at least 250 mg/dL per band, and that the system is sensitive to 2.0 mg/dL per band.

Comparison Studies

Patient samples were run for the correlation study done in conjunction with Helena's REP® 3 Auto-Flur Cholesterol system and the REP® Cholesterol Profile system. The range of total cholesterol for the samples was 115 mg/dL-311 mg/dL. All patients had triglyceride levels below 500 mg/dL. The following is the correlation data for individual bands on the two systems.

X = REP® Cholesterol Profile

Y = REP® 3 Auto Flur Cholesterol

n = 20

	Y =	R
HDL	0.804X +5.132	0.955
VLDL	1.361X + 2.335	0.932
LDL	1.166X - 18.772	0.884
All Bands	0.779X + 7.170	0.968

References to REP® Cholesterol refers to a system where the fractions are visible and reference to REP® 3 Auto Flur Cholesterol refers to laboratory testing of the present invention.[8]

8 As of the date the priority application was filed.

Claims

What is claimed is:

1. A reagent for staining lipoproteins for fluorometric analysis of electrophoretic bands, said reagent comprising at least one enzymic component, and an oxidizing agent component.

2. The reagent of claim 1, wherein said at least one enzymic component comprises at least two enzymes.

3. The reagent of claim 2, wherein one of said at least two enzymes is a Cholesterol Dehydrogenase.

4. The reagent of claim 3, wherein said Cholesterol Dehydrogenase is isolated from Nocardia sp.

5. The reagent of claim 3, wherein a second of said at least two enzymes is a Cholesterol Esterase.

6. The reagent of claim 5, wherein the oxidizing agent is a proton acceptor co-enzyme.

7. The reagent of claim 6, wherein said reagent further comprises an alkaline buffer.

8. The reagent of claim 7, wherein said proton-acceptor co-enzyme is nicotinamide adenine dinucleotide.

9. The reagent of claim 8, wherein said buffer has a pH of 7.5 to 9.5.

10. The reagent of claim 9, wherein the buffer is Tricine.

11. A method for fluorescent analysis of the cholesterol content of high density lipoproteins (HDL), low density lipoproteins (LDL), and very low density lipoproteins (VLDL) on a electrophoretic plate, said method comprising:

separating the lipoproteins electrophoretically;

staining the separated proteins with a reagent comprising at least one enzymic component and an oxidizing agent component; and

exciting the stained proteins with an appropriate wavelength of electromagnetic radiation; and

scanning the exited proteins with a densitometer with fluorometric capabilities.

12. The method of claim 11, wherein said at least one enzymic component comprises at least two enzymes.

13. The method of claim 12, wherein one of said at least two enzymes is a Cholesterol Dehydrogenase.

14. The method of claim 13, wherein said Cholesterol Dehydrogenase is isolated from Nocardia sp.

15. The method of claim 13, wherein a second of said at least two enzymes is a Cholesterol Esterase.

16. The method of claim 15, wherein the oxidizing agent is a proton acceptor co-enzyme.

17. The method of claim 16, wherein said reagent further comprises an alkaline buffer, and said proton acceptor co-enzyme is nicotinamide adenine dinucleotide.

18. The method of claim 17, wherein said buffer has a pH of 7.5 to 9.5.

19. The method of claim 18, wherein the buffer is Tricine.

20. The method of claim 19, wherein said method further comprises the step of drying the electrophoretic plate before staining the proteins, and the step of drying the electrophoretic plate after staining the proteins, but before scanning them.

Docket No.
5093

Declaration and Power of Attorney For Patent Application

English Language Declaration

As a below named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name,

I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

CHOLESTEROL SEPARATION AND FLUORESCENT ANALYSIS

the specification of which

(check one)

☐ is attached hereto.

☒ was filed on 23 July 1997 as United States Application No. or PCT International Application Number PCT/US97/13321

and was amended on ______________

(if applicable)

I hereby state that I have reviewed and understand the contents of the above identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, Code of Federal Regulations, Section 1.56.

I hereby claim foreign priority benefits under Title 35, United States Code, Section 119(a)-(d) or Section 365(b) of any foreign application(s) for patent or inventor's certificate, or Section 365(a) of any PCT International application which designated at least one country other than the United States, listed below and have also identified below, by checking the box, any foreign application for patent or inventor's certificate or PCT International application having a filing date before that of the application on which priority is claimed.

Prior Foreign Application(s)

(Number)	(Country)	(Day/Month/Year Filed)	Priority Not Claimed
PCT/US97/13321	PCT	23 July 1997	☐
			☐
			☐

I hereby claim the benefit under 35 U.S.C. Section 119(e) of any United States provisional application(s) listed below:

60/022,354	**24 July 1996**
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)
(Application Serial No.)	(Filing Date)

I hereby claim the benefit under 35 U. S. C. Section 120 of any United States application(s), or Section 365(c) of any PCT International application designating the United States, listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in the prior United States or PCT International application in the manner provided by the first paragraph of 35 U.S.C. Section 112, I acknowledge the duty to disclose to the United States Patent and Trademark Office all information known to me to be material to patentability as defined in Title 37, C. F. R., Section 1.56 which became available between the filing date of the prior application and the national or PCT International filing date of this application:

(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)
(Application Serial No.)	(Filing Date)	(Status) (patented, pending, abandoned)

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

POWER OF ATTORNEY: As a named inventor, I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith. *(list name and registration number)*

Jerold I. Schneider, Reg. No. 24,765
Aldo Noto, Reg. No. 35,628
John W. Ryan, Reg. No. 33,771
W. Robinson H. Clark, Reg. No. 41,530
John K. Harrop, Reg. No. 41,817
Larry Moskowitz, Reg. No. 42,911
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1-10

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2-10

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3-w

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4-w

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